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Edited by

Ray C. Friesner

The *Butler University Botanical Studies* journal was published by the Botany Department of Butler University, Indianapolis, Indiana, from 1929 to 1964. The scientific journal featured original papers primarily on plant ecology, taxonomy, and microbiology. The papers contain valuable historical studies, especially floristic surveys that document Indiana's vegetation in past decades. Authors were Butler faculty, current and former master's degree students and undergraduates, and other Indiana botanists. The journal was started by Stanley Cain, noted conservation biologist, and edited through most of its years of production by Ray C. Friesner, Butler's first botanist and founder of the department in 1919. The journal was distributed to learned societies and libraries through exchange.

During the years of the journal's publication, the Butler University Botany Department had an active program of research and student training. 201 bachelor's degrees and 75 master's degrees in Botany were conferred during this period. Thirty-five of these graduates went on to earn doctorates at other institutions.

The Botany Department attracted many notable faculty members and students. Distinguished faculty, in addition to Cain and Friesner , included John E. Potzger, a forest ecologist and palynologist, Willard Nelson Clute, co-founder of the American Fern Society, Marion T. Hall, former director of the Morton Arboretum, C. Mervin Palmer, Rex Webster, and John Pelton. Some of the former undergraduate and master's students who made active contributions to the fields of botany and ecology include Dwight. W. Billings, Fay Kenoyer Daily, William A. Daily, Rexford Daudenmire, Francis Hueber, Frank McCormick, Scott McCoy, Robert Petty, Potzger, Helene Starcs, and Theodore Sperry. Cain, Daubenmire, Potzger, and Billings served as Presidents of the Ecological Society of America.

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THE EFFECT OF LARGE AMOUNTS OF CERTAIN VITAMINS OF THE B GROUP ON THE GROWTH RATE AND MORPHOLOGY OF CERTAIN BACTERIA[1]

By ALICE JEANNE GILLUM

From the general literature on the subject of growth stimulants, it appears that large amounts of any growth stimulant will eventually cause a retardation of growth. The purpose of this study was to see what effects high concentrations of some of the vitamins of the B group have on the growth rate and morphology of a few of the common types of bacteria.

The few available papers dealing with vitamins of the B group are concerned with large amounts of nicotinic acid or nicotinamide. Kosar and Kasai (1) report marked retardation of growth when using 3,000, 5,000, and 10,000 gamma/ml of nicotinic acid and nicotinamide. Dorfman et al. (2) reported retardation of dysentery bacilli by concentrations of 3,000, 5,000, and 10,000 gamma/ml of nicotinic acid.

Other papers reporting similar retardations of growth with use of large amounts of nicotinic acid or nicotinamide are those of Koser and Baird (3) and Moller and Birkofer (4). Smaller amounts were found by Rosenfeld and Greene (5) to inhibit growth of Leptospira.

METHODS AND MATERIALS

Stock cultures of all organisms used were grown on Bacto-Nutrient Agar. This medium was also used in the poured plates for the plate counts. Media were adjusted to pH 7.2. A synthetic basal medium was used for the growth of the organisms during the experiments. Five cc. amounts of this medium were tubed and sterilized, and the vitamin solutions were added aseptically when desired. For certain organisms, additional nutrients were necessary to maintain growth. *Proteus vulgaris* required cystine, glutamic acid, and nicotinic acid; *Bacillus subtillis* required cystine and glutamic acid.

[1] A portion of a thesis submitted in partial fulfillment of the requirements for the Master of Science degree in the Division of Graduate Instruction, Butler University.

The preparation of the basal medium and additional nutrients is as follows:

Basal Medium (Koser and Kasai).

$(NH_4)_2HPO_4$	2.0 grams
KH_2PO_4	1.5 grams
NaCl	5.0 grams
$MgSO_4$	0.1 gram
Glucose	5.0 grams
Distilled water	1,000.0 ml.

pH 6.8 to 6.9 after autoclaving; must be readjusted after adding large amounts of nicotinic acid.

The following are added for *Proteus vulgaris* and *Bacillus subtilis* cultures:

Cystine	0.024 mg/ml
Glutamic acid	1.0 mg/ml

For *Proteus vulgaris* cultures:

Nicotinic acid	1 gamma/ml

Vitamins

The following vitamins were supplied, in pure form, by the Eli Lilly Company of Indianapolis, Indiana:

Riboflavin
Nicotinamide
Nicotinic acid
Pyridoxine hydrochloride
Thiamin hydrochloride

Sterilization of Vitamins

Maximum temperatures for heating of vitamins:

Riboflavin	527° F.
Nicotinamide	264.2-267.8° F.
Pyridoxine hydrochloride	320° F.
Thiamin hydrochloride	230° F.
Nicotinic acid	256° F.
Autoclave at 15 lbs.	248° F.
Autoclave at 0 lbs.	212° F.

The results of comparative tests showed no real difference in effect whether the vitamins were filtered or autoclaved (including intermittent sterilization). The vitamins were sterilized in dry form in the autoclave at correct temperature and pressure. Distilled water was boiled and autoclaved at 15 lbs. pressure for 20 minutes. The final pH of the distilled water was pH 7.0. Each vitamin was then dissolved in the previously prepared distilled water to give a specific concentration per ml. The correct amount of the vitamin-water solution was added to the tubed basal medium to give the concentrations desired.

Comparative tests showed the addition of largest amount of distilled water used in making proper concentration of vitamin had no effect on growth rate or morphology.

Procedure

The procedure followed in all experiments is as follows:

1. A 24-hour basal medium culture of the organism was obtained from a pure culture agar slant. All organisms used were Butler University stock cultures.

2. One loopful of this 24-hour basal medium culture was inoculated to each tube of basal medium.

3. The proper concentration of the vitamin was added to each tube.

4. Incubation at 37° C. (for all except *Bacillus subtilis,* which was incubated at 30° C.) for 16, 24, 48, and 72 hours.

5. Agar plates were poured for 16, 24, 48, and 72 hour counts. Dilution method was necessary in the case of high counts.

6. Slides were made at 16, 24, 48, and 72 hour intervals. Staining time was 1½ minutes.

7. Agar plates read by means of a Quebec "Dark Field" counter after 24 and 48 hours of incubation at 37° C. (30° C. for *Bacillus subtilis*).

8. The actual count was recorded and descriptions of the slides made in a record book.

Explanation of the Numbers Used in the Tables

The plate count method was used to estimate the count per ml of the basal medium.

The desired end was to obtain an estimate of the trend of growth rather than an actual recording of figures. For this reason, a conversion scale of from 0 to 100 was set up with each step interval equal to 40,000,000 cells, i.e., 0 means from 1 to 40,000,000 cells; 1 means from 40,000,000 to 80,000,000 cells; 2 means 80,000,000 to 120,000,000; etc. Thus, the higher the conversion scale figure, the higher the actual count. Growth rate was recorded on the charts in numbers of from 0 to 100 except where no growth occurred (recorded as NG). In this manner, all major changes in growth rates were shown without the confusion of large figures.

RESULTS

The general trend found with all vitamins used is that of increasing retardation of growth rate with an increase of vitamin concentration from the 1,000 gamma/ml level to the 10,000 gamma/ml level. There is a considerable tolerance to high concentrations of vitamins. A concentration of 1,000 gamma/ml showed a relatively low degree of effect on growth rate or morphology, while a concentration of 10,000 gamma/ml showed a maximum inhibition of growth and changes in morphology. The cells became swollen, granular, or beaded in appearance, taking the stain very faintly.

Total inhibition of growth occurred in the following cases: nicotinamide used with *P. vulgaris* and *Ps. aeruginosa* (table 5)[1] at the 10,000 gamma/ml level; nicotinic acid (table 6) used with *P. vulgaris* at the 10,000 gamma/ml level; pyridoxine hydrochloride (table 7) used with *P. vulgaris* at the 5,000 and 10,000 gamma/ml level in the 72-hour growth phase and with *Ps. aeruginosa* at the 3,000, 5,000 and 10,000 gamma/ml level; and thiamin hydrochloride (table 9) used with *Ps. aeruginosa* at the 10,000 gamma/ml level.

Cultures which have a conversion scale reading of 0 (definite inhibition but not total inhibition) are more numerous than those showing no growth. The 0 figure is found in the following cases: nicotinamide (table 5): with *P. vulgaris* at the 1,000, 3,000, and 5,000 gamma/ml level in the 16-hour phase of growth and also for the 5,000 gamma/ml in the 24-hour phase of growth; nicotinic acid (table 6): with *P. vulgaris* at the 3,000 and 5,000 gamma/ml in the 16-hour

[1] Table numbers are those of the original thesis and are not reproduced in the present paper. They are available on loan from the Butler University library.

phase and also for the 5,000 gamma/ml in the 24-hour phase; pyridoxine hydrochloride (table 7) : with *B. subtilis* at the 1,000 gamma/ml in the 16-hour phase and for the 3,000, 5,000, and 10,000 gamma/ml in all phases used, and with *P. vulgaris* at the 1.000 gamma/ml level in the 16-hour phase, at the 3,000 gamma/ml level in the 16- and 24-hour phases, and at tbe 5,000 and 10,000 gamma/ml level in the 16-, 24-, and 48-hour phases; riboflavin (table 8) : with *P. vulgaris* at the 1,000, 3,000, and 5,000, and 10,000 gamma/ml level in the 72-hour phase and also for the 10.000 gamma/ml level in the 16-hour phase; and finally, thiamin hydrochloride (table 9) : with *B. stubtilis* at the 3,000 and 5,000 gamma/ml level in the 16- and 24-hour phases and at the 10,000 gamma/ml level in all phases used, with *P. vulgaris* at the 3,000 gamma/ml level in the 16- and 24-hour phases and at 5,000 and 10,000 gamma/ml levels in all phases, and with *Ps. aeruginosa* at the 5,000 gamma/ml level in the 16-hour phase.

In many cases the initial inhibition at a certain concentration of vitamin will be almost overcome by the time the 72-hour phase is reached. Reference may be made to the following cases: nicotinamide with *P. vulgaris* and *Ps. aeruginosa* (table 5) ; nicotinic acid with *P. vulgaris* (table 6) ; pyridoxine hydrochloride with *B. subtilis* and *P. vulgaris* (table 7) ; riboflavin with *Ps. aeruginosa* (table 8) ; and thiamin hydrochloride with *B. subtilis, E. coli,* and *Ps. aeruginosa* (table 9).

In certain cases the growth at a later growth phase will be lower than at the early growth phases. For example, riboflavin with *B. subtilis* and *P. vulgaris,* (table 8). Riboflavin is very difficult to keep in solution in the amounts used and this may have some bearing on the results.

All organisms used in this experiment need no preformed vitamins (according to latest available data) except *P. vulgaris,* which must have nicotinic acid for growth. In spite of this, some organisms showed increased growth at higher concentrations of the vitamins. For example, nicotinamide with *B. subtilis* at the 10,000 gamma/ml level in 72 hours (table 5) ; nicotinic acid with *Ps. aeruginosa* at 1,000 gamma/ml level in 24 hours (table 6) ; riboflavin with *B. subtilis* at the 1,000 gamma/ml level in all growth phases and at the 3,000 gamma/ml level in the 16-, 24-, and 48-hour phases, with *E. coli* at 1,000, 3,000, and 5,000 gamma/ml in the 48-hour phase, with *Ps.*

aeruginosa at the 1,000, 3,000 and 5,000 gamma/ml levels in the 16-hour phase (table 8); and thiamin hydrochloride with *B. subtilis* at the 1,000 gamma/ml level in the 16- and 24-hour phases, with *P. vulgaris* at the 1,000 gamma/ml level in all growth phases (table 9).

In a few cases the growth rate went up for several growth phases and later came down again as in the case of riboflavin with *B. subtilis* and *P. vulgaris* in table 8. *B. subtilis* (table 1) appeared to be most sensitive to pyridoxine hydrochloride and thiamin hydrochloride. This organism seemed to be least sensitive to nicotinic acid and nicotinamide. Riboflavin was intermediate in effect.

E. coli (table 2) was most sensitive to nicotinamide and pyridoxine hydrochloride. Riboflavin affected this organism the least, while thiamin hydrochloride and nicotinic acid had a somewhat intermediate effect. *P. vulgaris* (table 3) was most sensitive to nicotinamide and nicotinic acid. Thiamin hydrochloride and pyridoxine hydrochloride caused a considerable effect, also. Riboflavin caused the least effect. *Ps. aeruginosa* (table 4) was most sensitive to pyridoxine hydrochloride with nicotinamide and thiamin hydrochloride causing a considerable effect. Riboflavin was the intermediate in effect, and nicotinic acid caused the least effect.

In order of decreasing inhibition, the vitamins affected the organisms in the following manner: (table 5) nicotinamide: *Ps. seruginosa, P. vulgaris, E. coli,* and *B. subtilis;* (table 6) nicotinic acid: *P. vulgaris, E. coli, Ps. aeruginosa,* and *B. subtilis;* (table 7) pyridoxine hydrochloride: *Ps. aeruginosa, P. vulgaris, B. subtilis,* and *E. coli;* (table 8) riboflavin: *P. vulgaris, B. subtilis, Ps. aeruginosa,* and *E. coli;* and table 9) thiamin hydrochloride: *Ps. aeruginosa, B. subtilis, P. vulgaris,* and *E. coli.*

These foregoing comparisons of relative effect were made on a rough basis, taking into account the amount of growth at the 0 level of concentration as compared with the 10,000 gamma/ml concentration. Taking a broad view, *Ps. aeruginosa* and *P. vulgaris* seemed to be more sensitive to the vitamins used in this experiment than did *E. coli* and *B. subtilis.* Pyridoxine hydrochloride and thiamin hydrochloride seemed to cause more inhibition, in general, than the other vitamins used.

As reported by Koser and Kasai (1), P. vulgaris needs nicotinic acid for growth. Initial experimentation, using the basal medium

plus cystine and glutamic acid in the required amounts (see Methods and Materials) when attempting to culture *P. vulgaris,* failed. Addition of 1 gamma/ml of nicotinic acid to the basal medium mentioned above supported growth quite well. The other organisms used grew well in the basal medium without the addition of preformed vitamins.

In order to check the effect of large concentrations of vitamins upon the morphology of the cells, slides were made from all concentrations at each growth phase. On examination of the slides, it was found that the cultures which were inhibited in their growth by the excessive amounts of vitamin showed, on staining with crystal violet, swelling and irregular staining ("beaded" staining, faint staining and the like). This irregularity appeared mostly in the 10,000, somewhat in the 5,000, and rarely in the 3,000 gamma/ml concentration. The 1,000 gamma/ml concentration appeared to show none of the irregularities of staining or shape.

DISCUSSION

Considering the minute amounts of the vitamins which are needed for optimal growth, it is rather surprising to find such a tolerance to high concentrations. 1,000 gamma/ml has really little effect on growth rate or morphology. Concentrations of 3,000, 5,000, and 10,000 gamma/ml must be reached before growth is retarded to any degree. A concentration of 10,000 gamma/ml seems to cause the most marked inhibition, many times causing complete inhibition of growth.

It will be noted that any initial difference in growth rate between the 0 and 1,000 gamma/ml concentrations tends to be overcome in later growth phases. This is also found in some of the 3,000 gamma/ml concentrations and less often with the 5,000 gamma/ml concentrations. In the 10,000 gamma/ml concentration the initial difference in growth rate is rarely overcome.

In the case of riboflavin with *B. subtilis* and of thiamin hydrochloride with *P. vulgaris,* the 1,000 gamma/ml concentration showed more growth than the 0 concentration even through the 72-hour growth phase. In several other cases, the initial growth rate seemed to be greater with the 1,000 gamma/ml concentration than with the 0 concentration, but this difference was overcome by the 72-hour growth phase. In the 10,000 gamma/ml concentration of nicotina-

mide with B. subtilis the 72-hour growth rate is greater than in the 0 concentration.

It should be remembered that the environmental conditions of this experiment are rather unbalanced. The cells are supplied large doses of one vitamin while others must be synthesized. Koser and Kasai (1) noted that, in the case of large amounts of nicotinic acid and nicotinamide with casein hydrolyzate, the inhibition was less marked. The same authors also found that yeast extract would nullify the effects of large amounts of nicotinic acid and nicotinamide. This could very well be the case with the other vitamins used in this experiment as it is well known that, when the environmental conditions are less satisfactory, multiplication is difficult and slow, and that changes in the form of bacteria may take place (swelling, faint staining, and granulation).

Hypothetical explanations of the inhibitory effects found in these experiments must surely rest on cellular physiology. It has been suggested in the general literature on cell physiology that alteration in the supply of growth factors will generally have decided repercussions in regard to cell metabolism. Cellular metabolism, depending as it does on a series of enzyme systems, is of necessity affected by factors which affect enzymes. Also, enzymes, being protein complexes, are chemically and physically unstable and sensitive to those factors to which protein complexes are sensitive, such as excessive concentration of any component of the substrate. Stated in another way, we might say that the actual effectiveness of the inhibitor corresponds to its ability to interfere with a chain of synthetic reactions in the cell. So we might conceivably imagine an essential metabolite which stimulates growth at certain levels for certain organisms, becoming, in a sense, an "anti-metabolite" when it reaches higher levels of concentration. "Anti-metabolite" is used here not in the strict chemical sense of a structural analogue but in the broader sense of any substance acting against normal metabolic processes. Viewed in this light. it does not seem so surprising that a growth stimulant, such as a vitamin, when used in large concentrations, should become a growth inhibitor. In fact, it is surprising to find a tolerance to such high concentrations of vitamin. For instance, 0.1 gamma/ml of nicotinamide is said to be sufficient for optimal growth of *P. vulgaris* (Koser and Kasai (1)) while complete inhibition of growth in this experiment did not take place until 10,000 gamma/ml was reached.

All organisms used were known to grow in the basal medium without the addition of preformed vitamins, except *P. vulgaris* which needs nicotinic acid to support growth. In spite of this fact, some of the organisms did show apparent growth stimulation at higher concentrations of vitamins. The probable explanation of this behavior is the fact that these are undoubtedly different strains of the organisms than those for which the original nutritional requirements were worked out. Koser and Kasai (1) report that different strains of *P. vulgaris* proved to have different sensitivity to large amounts of nicotinic acid and nicotinamide. The fact that in most cases no vitamin was used in the first series and 1,000 gamma/ml of vitamin was used as the first concentration would tend to mask the actual concentration at which growth was stimulated. In the cases, where there was apparent growth stimulation at higher than 1,000 gamma/ml level and at sporadic time intervals, some other factor may be responsible for the increased growth shown. Of course, in using the plate count method, one must always take into account the inherent error of the method when sporadic differences of growth occur, even when these are average plate counts. In other words, even using a conversion scale which tends to minimize such inherent error and even when using an average of several experiments, the factor of inherent error in the plate count and in the procedure in general still remains. However, in spite of these unavoidable errors, the results are reliable enough to give us a general trend of growth rate.

It is of importance to note what other workers have found in the few available reports in comparison to the results obtained in this paper. All of the reports deal with nicotinic acid and nicotinamide. For *P. vulgaris,* Moller and Birkofer (4) found that nicotinic acid caused marked reduction of growth or complete inhibition at 0.2 to 0.26 molar (about 30,000 gamma/ml level) concentrations and nicotinamids at 0.056 to 0.066 molar (about 6,000 gamma/ml) concentrations. Koser and Kasai (1) found that nicotinic acid caused marked inhibition at the 10,000 gamma/ml level and the same for nicotinamide. The present results show complete inhibition with nicotinamide at the 10,000 gamma/ml level and the same for the nicotinic acid. Moller and Birkofer (4) used 24- or 48-hour counts in contrast to the 16-, 24- 48-, and 96-hour counts of Koser and Kasai (1) and the 16-, 24-, 48-, and 72-hour counts of the present study.

For *E. coli,* Koser and Kasai (1) find markedly lower growth rate with nicotinic acid at the 10,000 gamma/ml level and very little growth with nicotinamide at the same level up through the 48-hour phase. The present study shows lower growth rate with nicotinic acid at 10.000 gamma/ml level and a markedly lower growth rate with nicotinamide at 10,000 gamma/ml level at all time intervals.

For *B. subtilis,* Koser and Kasai (1) found inhibition for the 16-hour phase at 1,000 gamma/ml and for longer intervals at the 10,000 gamma/ml level. This is for both vitamins. The present study shows *B. subtilis* not to be so sensitive to either vitamin.

The other reports mentioned above reported estimates of comparative growth on the basis of turbidity rather than by actual plate count, hence more detailed comparison of the findings can not be made.

CONCLUSIONS

1. In low concentrations, certain vitamins of the B group have little effect on *Ps. aeruginosa, B. subtilis,* or *E. coli.*

2. Small amounts of added nicotinic acid have a stimulating effect on *P. vulgaris.*

3. Massive doses of certain vitamins of the B group have a retarding effect on growth for the organisms used.

4. There is increasing retardation of growth rate with an increase of vitamin from the 1,000 gamma/ml concentration to the 10,000 gamma/ml concentration when the cultures are grown in a synthetic medium.

5. When there is marked inhibition of growth, the cells become swollen, granular, or beaded and take the stain very faintly.

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